Hyper-N-glycosylated SAMD14 and neurabin-I as driver autoantigens of primary central nervous system lymphoma

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Abstract: To address the role of chronic antigenic stimulation in primary central nervous system lymphoma (PCNSL), we searched for autoantigens and identified sterile -motif domain containing protein 14 (SAMD14) and neural tissue-specific F-actin binding protein I (neurabin-I) as autoantigenic targets of the B-cell receptors (BCRs) from 8/12 PCNSLs. In the respective cases, SAMD14 and neurabin-I were atypically hyper–glycosylated (SAMD14 at ASN339 and neurabin-I at ASN1277), explaining their autoimmunogenicity. SAMD14 and neurabin-I induced BCR pathway activation and proliferation of aggressive lymphoma cell lines transfected with SAMD14- and neurabin-I-reactive BCRs. Moreover, the BCR binding epitope of neurabin-I conjugated to truncated exotoxin-killed lymphoma cells expressing the respective BCRs. These results support the role of chronic antigenic stimulation by posttranslationally modified central nervous system (CNS) driver autoantigens in the pathogenesis of PCNSL, serve as an explanation for their CNS tropism, and provide the basis for a novel specific treatment approach.

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Hyper N-glycosylated SAMD14 and Neurabin-I as Driver CNS Autoantigens of PCNSL

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In Memoriam to Michael Pfreundschuh

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Keypoints

- Two thirds of PCNSL have BCRs specific for SAMD14 and neurabin-I, two highly homologous CNS proteins.
- Both antigens were posttranslationally modified and induced a strong BCR pathway activation and proliferation.

Abstract

To address the role of chronic antigenic stimulation in PCNSL we searched for auto-antigens and identified SAMD-14 and neurabin-I as auto-antigenic targets of the B-cell receptors (BCRs) from 8/12 PCNSLs. In the respective cases SAMD14 and neurabin-I were atypically hyper-N-glycosylated (SAMD14 at ASN339 and neurabin-I at ASN1277) explaining their auto-immunogenicity. SAMD14 and neurabin-I induced BCR pathway activation and proliferation of aggressive lymphoma cell lines transfected with SAMD14 and neurabin-I reactive BCRs. Moreover, the BCR binding epitope of neurabin-I conjugated to a truncated pseudomonas exotoxin killed lymphoma cells expressing the respective BCRs. These results support the role of chronic antigenic stimulation by posttranslationally modified CNS driver auto-antigens in the pathogenesis of PCNSL, serve as an explanation for their CNS tropism and provide the basis for a novel specific treatment approach.

Introduction

PCNSL is a rare aggressive B-cell non-Hodgkin lymphoma affecting the brain, the leptomeninges, the spinal cord or the eyes. Recently high frequency of activating CD79b and MYD88 mutations linking the BCR and TLR9 pathways and high expression of PDL1 were reported.[1;2] The reason for the selective tropism in the CNS, which is traditionally regarded as an immune privileged site, is still unclear. The tetrad of overrepresentation of autoreactivity-linked VH4-34, persistent functional

variable region genes despite ongoing somatic hypermutation, the negativity for EBV, and the transcriptional upregulation of the NF-kB pathway suggest chronic B-cell receptor activation and prompted us to search for antigens of the BCRs from PCNSL.[3-6] The hypothesis of chronic BCR stimulation contributing to the malignant transformation of B cells is old [7] and several specific target antigens of BCR from various B-NHLs have been identified.[8-11] A recently published study proposed auto- and polyreactive BCRs in PCNSL. [12]

Methods

Patients

The study had been approved by the local ethics committee (Ärztekammer des Saarlandes). Snap-frozen PCNSL specimens from 21 HIV-negative patients were obtained from the institutes of pathology and neuropathology of the universities of Saarland, Kiel, Würzburg and Erlangen. Pairs of serum and CSF samples from a second series of 22 PCNSL patients were obtained from the Department of neurology of Tübingen University. Additional sera and whole blood samples were obtained from the Departments of Hematology & Oncology of Freiburg and Saarland Universities. Sera from patients with secondary CNS manifestations or with testicular manifestations of DLBCL treated within the RICOVER-60 [13] trial of the DSHNHL served as a control. Blood Samples of multiple sclerosis were obtained from the department of neurology of Saarland Medical school. Cryospecimen of primary testicular lymphoma were obtained from the institutes of pathology of the universities of Würzburg and Ulm. Recombinant Fabs of other B cell neoplasia as controls for PCNSL were constructed in cooperation with the Secnkenberg institut of pathology of the Goethe university in Frankfurt a.M.

Ig variable region gene PCR

Fragments of PCNSL cryosections were digested with 2 µl of proteinase K (Roche PCR grade) for 4h at 55°C followed by heat-inactivation at 95°C for 10min. Semi-nested PCRs for VH, Vκ and Vλ genes were performed (30 and 44 cycles, respectively) using Expand High Fidelity PCR kit (Roche) as described by Küppers *et al.*[14;15] The resulting variable region genes were sequenced and analyzed with IMGT-V-Quest for functionality, V(D)J segment usage, and mutations. Whenever both a functional Ig heavy and light chain variable region gene were obtained, these genes were cloned into a modified pCES vector for the expression of recombinant BCRs as Fab fragments.[16] Fabs were expressed and purified as described previously.[17] Variable gene fragments were re-extended at the 5′ and 3′ ends according to the proper immunoglobulin germline genes. Complete V genes were inserted via ApaLI and XhoI for Vκ or Vλ in front of a κ- or λ-constant region gene, respectively and via *Nco*I and *BstE*II for VH in front of a γ-1 constant region gene into a modified pCES-1 vector for expression of the Fab fragment.[16] Fabs were expressed in *E. coli* TG1 strain and purified as described previously. [16;18;19]

Screening for antigenic BCR targets

Pooled recombinant PCNSL-BCRs (each at a concentration of 10 µg/ml) were screened on protein macroarrays representing clones of UniPEx human cDNA expression libraries expressed in *E. coli* (Bioscience, Dublin, Ireland) as described before. [10;11;20]

ELISA for BCR and serum antibody reactivity against SAMD14 and neurabin-I

SAMD14 and neurabin-I were recombinantly expressed with a C-terminal FLAG tag in HEK293 under the control of a CMV promoter (pSFI). Total cell extracts were bound at a concentration of 10 µg/ml to Nunc Maxisorb plates pre-coated overnight at 4°C with murine anti-FLAG antibody at a dilution of 1:2,500 (v/v; Sigma, Munich). Blocking was performed with 1.5% (w/v) gelatin in TBS and washing steps were performed with TBS-Tx [TBS, 0.1% (v/v) Tx100]. Individual recombinant Fabs (10 µg/ml), sera (1:100) and CSF (1:50) were used. ELISAs were conducted according to standard protocols with biotinylated goat anti-human IgG (heavy and light chain), goat anti-human IgA and anti-human IgM (all Dianova), all at dilutions of 1:2,500, or subclass-specific sheep anti-human IgG1, IgG2, IgG3 and IgG4 (Binding Site, Birmingham, UK) at dilutions of 1:5,000. Following this, correspondent biotinylated secondary antibodies were used for the immunoassays for IgG subclasses. For detection, peroxidase-labeled streptavidin (Roche) was used at a dilution of 1:50,000.

Identification of the BCR-binding epitope of SAMD14 and neurabin-I

For the determination of the binding region of SAMD14 and neurabin-I, recombinant fragments of different lengths from both antigens were constructed with C-terminal FLAG tags and expressed as described above.

Western blots of SAMD14 and neurabin-I

Lysates of PCNSL tissue specimens, whole peripheral blood cells or LCLs were loaded and separated in a 10% SDS-PAGE and transferred to PVDF membrane using a transblot semi-dry transfer cell (Bio Rad). After blocking overnight at 4°C in PBS/10% non-fat dry milk, transferred proteins were incubated with recombinant SAMD14/neurabin-I reactive BCRs at a concentration of 2 µg/ml for 1h at room

temperature or with rabbit anti N-terminus neurabin-I antibody (ABIN 1108406) at 1:500 for 1h or with murine anti-FLAG-antibody at 1:2,000 for 1h, followed by 1h incubation at room temperature with murine anti-His antibody at 1:2,000 (Quiagen), with HRP-labelled anti mouse IgG antibody (Biorad) for His6-tagged PCNSL-derived recombinant BCRs, with goat HRP-labelled anti-rabbit antibody (Bio Rad) at 1:3,000 for the anti-neurabin-I antibody, and by HRP-labelled anti-mouse-IgG antibody at 1:3,000 for Western blots with point-mutated FLAG-tagged full-length fragments of SAMD14 and neurabin-I and the chemiluminescence reagent (NEB) was used for immunoblot detection.

Moreover, SAMD14 and neurabin-I from >80 established cell lines were screened by Western blot for N-hyperglycosylation.

Deglycosylation of SAMD14 and neurabin-I

Equal amounts of denatured lysates from PCNSL specimens were treated with specific exo- or endoglycosidases, i.e. PNGase-F, o-Glycosidase, α-2(3,6,8,9)-neuraminidase, position-specific β-1-4-galactosidase or β-N-acetylglucosaminidase (EDEGLY-Kit, Sigma).

Site-directed mutagenesis

Using QuickChange II Site-Directed Mutagenesis Kit (Stratagene) and SAMD14 and neurabin-I full-length FLAG-tagged DNA fragments, several candidate N-glycosylation sites within or adjacent to the BCR binding epitopes were mutagenized after prediction of the most likely glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/). In each case the amino acid asparagine (N) was replaced by glutamine (Q), disabling N-glycosylation. This exchange was

performed for SAMD14 with N339Q and N372Q and for neurabin-I with N1208Q, N1258Q, N1277Q, N1289Q and N1301Q. These FLAG-tagged mutants were cloned into pRTS and expressed in LCLs derived of patients with N-hyperglycosylated SAMD14 and neurabin-I (#20) and patient with wt SAMD14 and neurabin-I (#21).

Lymphoblastoid cell lines

Lymphoblastoid cell lines (LCLs) were established by infection of PBMCs with EBV as described before.[21]

Expression of SAMD14/neurabin-I, MAZ antigens and respective immunotoxins

BCR-binding epitopes of neurabin-I, SAMD14 and MAZ as a control were recombinantly expressed with C-terminal FLAG tags in HEK293 cells.[11] Immunotoxins consisting of the BCR-binding epitope of neurabin-I (amino acids 1226-1251) conjugated to *pseudomonas* exotoxin A were recombinantly expressed in *E. coli* BL21 as described before by others.[22]

Expression of SAMD14/neurabin-I reactive BCR in OCI-Ly3, TMD-8 and U2932

As no EBV-negative PCNSL cell line exists to the best of our knowledge, the DLBCL ABC type cell lines OCI-Ly3, TMD-8 and U2932 were chosen as model cell lines. OCI-Ly3 harbors mutated MYD88 and CARD11, TMD-8 mutated MYD88, mutated CD79B, but wild-type CARD11, U2932 mutated TAK1, but wild-type CARD11 and MYD88.[23-26] Each cell line was transfected with a modified pRTS-1 expression vector with a variable region heavy chain and constant regions Cγ1-Cγ4 with membrane coding exons TM1 and TM2 for the transmembrane region and the cytoplasmic tail followed by a 2A sequence and the light chain variable region and light chain constant region gene inserted via SfiI sites.[27] Both VH and Vk were derived from the

SAMD14/neurabin-I reactive PCNSL-BCR from patient #18 or as a control from a patient with CLL and a BCR directed against MAZ[11]. Transfection of OCI-Ly3, TMD8 and U2932 cells was performed on ice after 3 washing steps with RPMI at a cell density of 2 x 10E7/ml in RPMI without FCS. 2 x 10E6 cells were transfected with 5 µg plasmid DNA by electroporation using Gen Pulser (Biorad) with a 0.2 cm cuvette at 140V with 30 msec pulses. Subsequently, cells were immediately put on ice again and cultured in RPMI-1640 medium supplemented with 20% FCS. Cell lines stably expressing surface BCR with reactivity against SAMD14/neurabin-I were selected with hygromycin 250 µg/ml.[28] Successful transfection was verified by variable region gene PCRs, by Western blot and flow cytometry of the FLAG-tagged recombinant BCRs.

Proliferation and BCR pathway activation assays

For Western blot analysis of the BCR pathway activation of transfected OCI-Ly3, cells either expressing a BCR with reactivity against SAMD14/neurabin-I or a different antigen, respectively, 1x10E6 cells were incubated with no antigen, neurabin-I/SAMD14 at 5 µg/ml, MAZ at 5 µg/ml or anti-IgM at 1 µg/ml. For Western blot analysis of the BCR pathway activation of transfected TMD8 and U2932 cells either expressing recombinant BCR with reactivity against SAMD14/neurabin-I by Doxycyclin induction or not expressing recombinant BCR by lack of Doxycyclin induction, 1x10E6 cells were incubated with no antigen, neurabin-I/SAMD14 at 5 µg/ml, LRPAP1 at 5 µg/ml or anti-IgM at 1 µg/ml. As primary antibodies rabbit antibodies against pTyr525/526 SYK diluted 1:2000, pTyr759 PLCγ2 diluted 1:1000, pTyr223 BTK diluted 1:1000 and pTyr96 BLNK diluted 1:1000 (B cell signaling sampler kit, CST, Massachusetts, USA), rabbit antibody against actin diluted 1:2000 (Sigma) and murine antibody against c-Myc at a concentration of 1µg/ml (Santa

Cruz) were used, followed by washing steps and incubation with POX-conjugated anti rabbit or anti mouse antibodies diluted at 1:3000.

For the analysis of cytoplasmatic calcium changes by flow cytometry a FACS Canto analyzer was used and Fluo-4/AM dye. Transfected OCI-Ly3 cells either expressing BCRs with reactivity against SAMD14/neurabin-I or against MAZ [11] were resuspended in calcium- and magnesium-free PBS, and loaded with Fluo-4/AM dye (final concentration 2 µM, Invitrogen) for 30 min at room temperature as described before.[11] Antigen was added followed by flow cytometry of the cells. Ionomycin (10 ng/µl, Sigma-Aldrich) was used as a positive control for the release of calcium from internal stores. Cytoplasmatic calcium levels were repeatedly analyzed immediately after adding the antigen to the dye-loaded cells and mixing.

Cell proliferation was determined by a non-radioactive assay (EZ4U, Biomedica) according to the manufacturer's instructions In short, $4x10^4$/ml OCI-Ly3 cells expressing BCRs with reactivity against SAMD14/neurabin-I or MAZ were seeded in 200µl cell cultures. To detect possible quantitative differences between the recombinantly expressed epitope regions of neurabin-I or MAZ, both were used at a concentration of 1 µg/ml. After 24 h incubation at 37ºC, 20 µl of chromophore substrate were added to each well.

Cytotoxicity, apoptosis and dye exclusion assays

For the analysis of the direct cytotoxic effects of immunotoxins an LDH release assay was used. 5x10E3/well OCI-LY3, TMD8 or U2932 cells stably transfected with Doxycycline-inducible expression of a SAMD14/neurabin-I-reactive BCR were incubated with neurabin-I-ETA' (5 µg/ml), MAZ-ETA' (5 µg/ml) or no immunotoxin. Percent specific lysis was determined as (experimental lysis minus spontaneous lysis)/(maximum lysis minus spontaneous lysis) × 100. Maximum lysis was

253 determined by adding 10% Triton X-100. Lactate dehydrogenase (LDH) was
254 measured according to the protocol of the LDH assay kit (Roche, Mannheim,
255 Germany). ELISA read out was done using a Victor II (PerkinElmer, Rodgau,
256 Germany).

257 For the analysis of apoptosis, 5x10E5 cells/ml suspension of OCI-LY3 cells stably
258 transfected to express either a BCR with reactivity against the SAMD14/neurabin-I or
259 against MAZ [11] were treated with neurabin-I/ETA', MAZ/ETA' (both at 0.5 µg/ml) or
260 staurosporin (1 µg/ml) for 24h at 37°C, 5% CO_2. Following the incubation, the cells
261 were washed twice with PBS and resuspended in 500 µl binding buffer. 5 µl of
262 AnnexinV-FITC and 10µl of propidium iodide were added to each cell suspension
263 and incubated for 10 min at room temperature, followed by analysis by FACS Canto.
264 In addition, the effects of the immunotoxins were measured by trypan blue assays at
265 0, 24 and 48 hours.

266

267 ## Results

268 **SAMD14 and neurabin-I as antigenic targets of BCRs from PCNSL**

269 The median age of the 21 patients from whom PCNSL specimens were obtained was
270 64 (range: 36 to 77) years. Functional Ig heavy and light chain genes were
271 successfully amplified from 12 PCNSL patients, including one case with two
272 functional heavy chain genes (case 3), and two cases with two functional light chain
273 genes (case 15 and 17). Four of the 12 (33%) patients had VH gene segments of
274 VH4-34. All cases with amplified V region genes were somatically mutated: one case
275 carried an unmutated VH gene, but a mutated Vk segment; two other cases carried
276 unmutated Vk genes, but mutated VH genes (supplementary table 1).

The screening of the protein macroarrays representing 7,390 distinct human proteins for reactivity with 12 pooled recombinant PCNSL-derived BCRs identified one candidate autoantigen with a strong reactivity on the Unipex1 membrane: an expression clone of SAMD14 variant 2 spanning from amino acids 192 to 417 (Supplementary Figure 1). No reactivities were detected on the Unipex2 membrane. An ELISA with recombinant SAMD14 variant 2 expressed with a C-terminal FLAG-tag in HEK293 confirmed SAMD14 as the only target antigen of 8/12 (67%) PCNSL-BCRs (Figure 1A).

None of the recombinant control BCRs derived from 12 peripheral DLBCL, 19 MCL, 31 FL and 15 NLPHL cases reacted against SAMD14 (Supplementary Figures 2 and 3). Western blot of denatured PCNSL lysates with the recombinant BCRs as primary antibodies confirmed reactivity against SAMD14, a protein of 45 kDa. An additional band of approximately 140 kDa was observed (Figures 1B - D). To investigate if this was a hypothetical polymer of SAMD14 or the highly homologous SAM domain of neurabin-I, the Western blot was stripped and repeated with an anti-neurabin-I antibody reactive against the non-homologous N-terminus of neurabin-I and not the homologous SAM domain. This identified neurabin-I as a second target containing a highly homologous SAM domain (Figure 1C). ELISAs with fragments of different lengths of SAMD14 variant 2 and of neurabin-I identified an epitope spanning from amino acid 274 to 417 of SAMD14 variant 2 and an epitope spanning from amino acid 995 to 1345 of neurabin-I (supplementary figures 4 and 5) as the BCR-binding region. In further analysis for SAMD14 amino acids 306 to 324 and for neurabin-I amino acids 1226 to 1251 were identified as the regions with the highest affinity. No reactivities of the PCNSL-BCRs were detected on an infectious disease epitope microarray.

Hyper-N-glycoslyation of SAMD14 and neurabin-I

Both SAMD14 variant 2 and neurabin-I had a higher molecular weight in PCNSL patients with reactive lymphoma BCRs compared to patients without reactive lymphoma BCRs and healthy controls (Figures 1C and 1E). Deglycosylation with PNGase F / ß-n-acetylglucosaminidase led to the disappearance of differences in molecular weights of both SAMD14 and neurabin-I between patients with and without SAMD14/neurabin-I-reactive lymphomas. Neither deglycosylation with O-glycosidase, α-2(3,6,8,9)-neuraminidase nor β-1-4-galactosidase (Figure 2A and 2B) induced this disappearance of differences in molecular weights. In 7/15 PCNSL patients with sufficient material for Western blot analysis, hyperglycosylated SAMD14 and neurabin-I were detected in cryosections and also (yet only weakly) in the peripheral blood from those patients of whom the latter was available. The hyperglycosylated SAMD14 and neurabin-I isoforms were also weakly expressed in monocytes, lymphocytes and granulocytes from PCNSL patients with SAMD14/neurabin-I-reactive BCRs (supplementary figure 6). Hyperglycosylated SAMD14/neurabin-I was not observed in the peripheral blood cell lysates from 400 healthy controls, 50 residents of nursery homes, 20 patients with multiple sclerosis, nor in cryospecimen of four cases of testicular lymphoma, nor in any of >80 established cell lines derived from human malignancies (data not shown). Subsequently, using patient-derived LCLs and point-mutated FLAG-tagged constructs of SAMD14 and neurabin-I, Asn 339 was identified to bear the hyperglycosylated Asn residue of SAMD14, and Asn 1277 of neurabin-I (Figure 2C). These glycosylation sites consisted of atypical N-L-E-Q.

Antibody reactivity against SAMD14 and neurabin-I

SAMD14/neurabin-I autoantibodies were frequently detected by ELISA in the sera and CSF from patients with PCNSL. The sera and CSF from 8/22 (36.4%) patients from a cohort different from the one from whom the PCNSL specimens were obtained contained anti-SAMD14/neurabin-I antibodies (supplementary figure 7) as was the case with 10 sera from a third series of 39 patients with PCNSL, resulting in a total of 18/61 (29.5%) serum antibody-positive patients. Antibody serum titers ranged from 1:800 up to 1:3200 and CSF titers ranged from 1:800 up to 1:1600 (Table 1). The eight SAMD14/neurabin-I antibody-positive serum/CSF pairs were tested for their Ig classes and IgG subclasses. All of them were IgG, with 7/8 (87.5%) belonging to the IgG1 subclass (supplementary figure 8 and 9). There were no SAMD14/neurabin-I antibodies in the sera from 18 patients with secondary CNS manifestations of peripheral DLBCL, sera of 24 patients with DLBCL with testicular manifestation, 92 healthy controls and in the sera and CSF of 21 patients with multiple sclerosis (supplementary figures 10, 11, 12, 13 and 15).

BCR pathway activation and induction of proliferation by SAMD14 and neurabin-I

Western blot analysis of the BCR signaling pathway showed a strong activation following the addition of the cognate antigen neurabin-I to transfected TMD8, U2932 and OCI-Ly3 cells expressing a SAMD14/neurabin-I-reactive BCR as demonstrated by a strong upregulation of pTyr525/526 SYK, pTyr96 BLNK, pTyr759 PLCγ2 and pTyr223 BTK and a significant increase in MYC expression (Figure 3A and supplementary Figure 18). Furthermore, flow cytometric analysis of cytoplasmic calcium levels of the OCI-Ly3 cells transfected to express a SAMD14/neurabin-I-reactive BCR indicated a strong increase of cytoplasmatic calcium levels after incubation with SAMD14/neurabin-I, but not the control antigen MAZ (supplementary

Figure 18), which had been shown to be the antigenic target derived from the malignant cells of a patient with CLL [11]. In correspondence to this, incubation with the BCR-binding neurabin-I epitope resulted in an increased proliferation of the transfected U2932, TMD8 and OCI-LY3 cell lines expressing SAMD14/neurabin-I-reactive BCR (Figure 3b and supplementary Figure 18) in a tetrazolium formazan (EZ4U) assay.

BCR-mediated targeting of PCNSL by BCR antigen / drug conjugates

The transfected cell lines Ocy-Ly3, U2932 and TMD8 expressing a patient-derived BCR with specificity for SAMD14/neurabin-I were specifically killed by incubation with a neurabin-I-ETA' drug conjugate, while remaining unaffected when incubated with the same toxin conjugated to a irrelevant control antigens (MAZ or LRPAP1). The specific killing was demonstrated in an LDH release assay (figure 4A). As shown by trypan exclusion, 100% of the lymphoma cells expressing a BCR with specificity for SAMD14/neurabin-I were dead after 72 hours of incubation with the BAR-toxin conjugate (figure 4b). The toxic effect of the neurabin-I/ETA' was dose-dependant (Figure 4c). In accordance to this, an increase of apoptotic cells was detected after incubation with neurabin-I/ETA' in U2932, TMD8 and OCI-Ly3 cells expressing SAMD14/neurabin-I-reactive BCRs (Figure 4 d and e and supplementary Figure 19), as shown by an Annexin-V/PI assay.

Discussion

We identified the sterile α-motif domain containing protein 14 (SAMD14) and the highly homologous SAM domain of neural tissue-specific F-actin binding protein I (neurabin-I) [29] as the predominant antigenic targets of the BCR from PCNSLs. The specificity of the PCNSL BCRs was not only demonstrated by the negative reaction

of the PCNSL-BCRs with the remaining >7000 other proteins represented in the macroarray used for screening, but also by the absence of any reactivity against >200 pathogens including various bacterial, fungal, parasitic and viral pathogens. The recombinant BCRs in the present study were not reactive against galectin-3 (supplementary figure 17).[12] Moreover, SAMD14/neurabin-I are specific antigens of PCNSL-derived BCRs, because none of the BCRs derived from 12 DLBCL, 19 MCL, 31 follicular lymphoma and 15 NLPHL cases reacted with these proteins which are preferentially expressed in the CNS.

SAMD14 and neurabin-I share a highly homologous SAM domain, neurabin-I has a further SAM-like neurabin domain, and both SAMD14 and neurabin-I are primarily expressed in the CNS (www.proteinatlas.org and www.genevisible.com). The SAMD14 gene, located on chromosome 17q21.33, consists of 14 exons and a coding sequence of 417 amino acids. Little is known about the function of SAMD14. The gene coding for neurabin-I, also called protein phosphatase 1 regulatory subunit 9A (PPP1R9A) is located on chromosome 7q21.3 and has 29 exons with 5 different splicing forms with coding sequences from amino acids 1095 to 1374. Among other functions, neurabin-I is a regulator of protein phosphatase 1 (PP1) and binds specifically to F-actin from neural tissues. Overexpression was observed in the dorsolateral prefrontal cortex of bipolar disorder and is believed to interact with NMDA receptor signaling pathways that regulate the actin cytoskeleton and spines of dendritic cells.[30] Moreover, neurabin-I was found to be specifically overexpressed in hepatosplenic T-cell lymphoma.[31]

SAMD14 and neurabin-I were N-hyperglycosylated in all patients with PCNSL-derived BCRs with reactivity for these antigens, but not in any other patient or control. Posttranslationally modified antigens are a well-known phenomenon in autoimmunity

[17;32-35]. The associated autoreactive BCRs and antibodies are either specifically reactive against the posttranslationally modified autoantigenic isoforms,[17;33] while for other antigens the BCRs or paraproteins did not differentiate between the posttranslationally modified and the wild-type isoform.[10;34;36;37] The latter is also the case for the PCNSL-derived BCRs in this study, which reacted with both the normally glycosylated and the hyper N-glycosylated SAMD14 and neurabin-I isoforms. Peptides of hyper-N-glycosylated SAMD14/neurabin-I are probably specifically recognized by CD4+ T cells, which stimulate non-isoform-specific SAMD14/neurabin-I reactive B cells similar to hyperphosphorylated SLP2 in plasma cell disorders.[38] SAMD14/neurabin-I autoantibodies showed no light chain restriction, but were observed in high titers in serum and CSF, indicating two things: First, it suggested a partly intrathecal production, and second, they probably do not originate directly from the lymphoma cells, but from related polyclonal B cells with the same specificity that have undergone plasmablastic differentiation, in contrast to the IgM^+ and IgD^+ PCNSL cells, which characteristically stop differentiation before class switch.[39]

For SAMD14 the residue of ASN 339 and for neurabin-I the residue of ASN 1277 were identified as the sites of N-hyperglycosylation. Interestingly, these two N-glycosylation sites are made of atypical N-L-E-Q patterns, which might further trigger autoimmunity. Functional effects of the N-hyperglycosylation of both proteins are not clear and remain to be analyzed in future studies.

In the era of stereotactic biopsies expression cloning of recombinant BCRs from PCNSL tissue represents a particular challenge because of the rare availability of sufficient fresh tissue. Despite the cooperation of the major PCNSL centers in the German-speaking countries, many PCNSL specimens included in this study were rather old (the oldest more than 25 years) and it was impossible to obtain sera or

CSF from all patients of whom cryospecimens were analyzed. Therefore, for the analysis of serum and CSF samples, we had to turn to a second cohort of patients. The detection of polyclonal antibodies against SAMD14/neurabin-I, which were found in the sera and CSF of nearly one-third of patients in a second (independent) PCNSL validation cohort, supports the hypothesis that the malignant transformation evolves from a polyclonal B-cell response against the N-hyperglycosylated and primarily in the CNS expressed SAMD14 and neurabin-I autoantigens as a non-random early step in PCNSL, followed by transformation of a subset of SAMD14/neurabin-I-reactive B-cells into a malignant clone.

To investigate if the reactivity of the lymphoma BCRs against SAMD14 and neurabin-I continues to play a sustaining role for established aggressive lymphoma, three different ABC type DLBCL cell lines, which are biologically closely related to PCNSL and represent different mutational backgrounds,[23-26] were transfected to express a SAMD14/neurabin-I-reactive BCR, as no established EBV-negative PCNSL cell line exists so far. Addition of the antigen SAMD14/neurabin-I to this transfected cell lines resulted in a strong BCR pathway activation accompanied by elevation of cytoplasmatic calcium levels, a stronger expression of c-Myc and a significantly increased proliferation.

The results of this study support the hypothesis that activation of the lymphoma BCRs by their cognate CNS antigen produces a strong growth advantage even for the transformed aggressive lymphoma cells. This is also supported and might explain the persistence of functional BCRs in PCNSL despite ongoing somatic hypermutation. The proliferative effects of the chronic BCR activation by SAMD14 and neurabin-I together with the fact that both SAMD14 and neurabin-I are predominantly expressed in the CNS [29] serve also as an explanation for the CNS

tropism of the respective lymphomas (Supplementary Figure 20: scheme of suspected role of SAMD14/neurabin-I).

Finally, the specific reactivity of lymphoma BCRs from the majority of patients with PCNSL against SAMD14 / neurabin-I might provide the basis for a specific therapeutic approach that uses BCR antigens for reverse targeting (BARs). Since SAMD14/neurabin-I are cytoplasmic proteins, treatment strategies using SAMD14/neurabin-I as a targeting moiety for directing a toxic payload to tumor cells should not bind to cells expressing membrane receptors other than a BCR with specificity for SAMD14/neurabin-I. Because it is the major physiologic task of the BCR to bind and internalize its specific antigen, SAMD14/neurabin-I BARs were readily internalized (data not shown) and - when conjugated to a toxin - killed the lymphoma cells after release of the toxin (ETA) with 100% of the cells being dead after 72 hours as demonstrated by cytotoxicity and apoptosis assays (Figure 4b) and dye exclusion. In contrast to approaches that target the idiotypic region of the BCR and must be designed for each individual patient, due to the predominance of BCRs with specificity for SAMD14/neurabin-I in PCNSL, SAMD14/neurabin-I can be used as bait to target a majority of patients with PCNSL.[40;41] Beside drug-conjugates, the epitope regions of SAMD14 and neurabin-I could as well be conjugated to or used as the targeting moiety of CAR-T cells, thus binding to and activating T-cells against the BCR-expressing cells. Because approaches using the BCR antigen for targeting the malignant B cells have a higher specificity, they can be expected to be less toxic than the currently available bispecific T-cell engaging antibodies or CAR-T-cells with specificity for CD19.[41]

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Authorship

LT, KDP and MP designed the study. YJK, RMB, RB, SA, PM, SS, SH, MLH, VP, SW, MZ, AM, PR, MW, MS, WK, PH, AR, CM collected the cryopreserved PCNSL tissues, serum and CSF samples from patients with PCNSL, PTL, DLBCL or MS. MK, LT, ER, NF performed the cloning and recombinant expression of the immunoglobulins. NF performed the protein array experiments and deglycosylation experiments. ER performed the site-directed point mutagenesis. MK and ER performed sequencing. LT and MP are responsible for data analysis, interpretation of results and writing of the manuscript. All authors read and approved the manuscript.

Conflict of interest disclosures: The authors declare no conflict of interest. Saarland University has applied for a relevant patent.

References

1. Chapuy B, Roemer MG, Stewart C et al. Targetable genetic features of primary testicular and primary central nervous system lymphomas. Blood 2016;127:869-881.

2. Montesinos-Rongen M, Schmitz R, Brunn A et al. Mutations of CARD11 but not TNFAIP3 may activate the NF-kappaB pathway in primary CNS lymphoma. Acta Neuropathol. 2010;120:529-535.

3. Montesinos-Rongen M, Kuppers R, Schluter D et al. Primary central nervous system lymphomas are derived from germinal-center B cells and show a preferential usage of the V4-34 gene segment. Am.J.Pathol. 1999;155:2077-2086.

4. Thompsett AR, Ellison DW, Stevenson FK, Zhu D. V(H) gene sequences from primary central nervous system lymphomas indicate derivation from highly mutated germinal center B cells with ongoing mutational activity. Blood 1999;94:1738-1746.

5. Montesinos-Rongen M, Godlewska E, Brunn A et al. Activating L265P mutations of the MYD88 gene are common in primary central nervous system lymphoma. Acta Neuropathol. 2011;122:791-792.

6. Montesinos-Rongen M, Schafer E, Siebert R, Deckert M. Genes regulating the B cell receptor pathway are recurrently mutated in primary central nervous system lymphoma. Acta Neuropathol. 2012;124:905-906.

7. DAMESHEK W, SCHWARTZ RS. Leukemia and auto-immunization- some possible relationships. Blood 1959;14:1151-1158.

8. Stevenson FK, Smith GJ, North J, Hamblin TJ, Glennie MJ. Identification of normal B-cell counterparts of neoplastic cells which secrete cold agglutinins of anti-I and anti-i specificity. Br.J.Haematol. 1989;72:9-15.

9. Martin T, Weber JC, Levallois H et al. Salivary gland lymphomas in patients with Sjogren's syndrome may frequently develop from rheumatoid factor B cells. Arthritis Rheum. 2000;43:908-916.

10. Preuss KD, Pfreundschuh M, Ahlgrimm M et al. A frequent target of paraproteins in the sera of patients with multiple myeloma and MGUS. Int.J.Cancer 2009;125:656-661.

11. Zwick C, Fadle N, Regitz E et al. Autoantigenic targets of B-cell receptors derived from chronic lymphocytic leukemias bind to and induce proliferation of leukemic cells. Blood 2013

12. Montesinos-Rongen M, Purschke FG, Brunn A et al. Primary Central Nervous System (CNS) Lymphoma B Cell Receptors Recognize CNS Proteins. J.Immunol. 2015;195:1312-1319.

13. Boehme V, Schmitz N, Zeynalova S, Loeffler M, Pfreundschuh M. CNS events in elderly patients with aggressive lymphoma treated with modern chemotherapy (CHOP-14) with or without rituximab: an analysis of patients treated in the RICOVER-60 trial of the German High-Grade Non-Hodgkin Lymphoma Study Group (DSHNHL). Blood 2009;113:3896-3902.

14. Kuppers R, Zhao M, Hansmann ML, Rajewsky K. Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. EMBO J. 1993;12:4955-4967.

15. Kuppers R, Schneider M, Hansmann ML. Laser-based microdissection of single cells from tissue sections and PCR analysis of rearranged immunoglobulin genes from isolated normal and malignant human B cells. Methods Mol.Biol. 2013;971:49-63.

16. de Haard HJ, van NN, Reurs A et al. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J.Biol.Chem. 1999;274:18218-18230.

17. Preuss KD, Pfreundschuh M, Fadle N, Regitz E, Kubuschok B. Sumoylated HSP90 is a dominantly inherited plasma cell dyscrasias risk factor. J.Clin.Invest 2015;125:316-323.

18. Voswinkel J, Kerkdijk AJ, Mueller A et al. A novel system to test for specificity of B cell receptors from tissue of Wegener's granulomatosis patients. Clin.Exp.Rheumatol. 2008;26:S90-S96.

19. Hoogenboom HR, Griffiths AD, Johnson KS et al. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res. 1991;19:4133-4137.

20. Thurner L, Muller A, Cerutti M et al. Wegener's granuloma harbors B lymphocytes with specificities against a proinflammatory transmembrane protein and a tetraspanin. J.Autoimmun. 2011;36:87-90.

21. Neumann F, Wagner C, Preuss KD et al. Identification of an epitope derived from the cancer testis antigen HOM-TES-14/SCP1 and presented by dendritic cells to circulating CD4+ T cells. Blood 2005;106:3105-3113.

22. Nachreiner T, Kampmeier F, Thepen T et al. Depletion of autoreactive B-lymphocytes by a recombinant myelin oligodendrocyte glycoprotein-based immunotoxin. J.Neuroimmunol. 2008;195:28-35.

23. Compagno M, Lim WK, Grunn A et al. Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. Nature 2009;459:717-721.

24. Davis RE, Ngo VN, Lenz G et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature 2010;463:88-92.

25. Mondello P, Brea EJ, De SE et al. Panobinostat acts synergistically with ibrutinib in diffuse large B cell lymphoma cells with MyD88 L265 mutations. JCI.Insight. 2017;2:e90196.

26. Ngo VN, Young RM, Schmitz R et al. Oncogenically active MYD88 mutations in human lymphoma. Nature 2011;470:115-119.

27. Freitag J, Heink S, Roth E et al. Towards the generation of B-cell receptor retrogenic mice. PLoS.One. 2014;9:e109199.

28. Bornkamm GW, Berens C, Kuklik-Roos C et al. Stringent doxycycline-dependent control of gene activities using an episomal one-vector system. Nucleic Acids Res. 2005;33:e137.

29. Nakanishi H, Obaishi H, Satoh A et al. Neurabin: a novel neural tissue-specific actin filament-binding protein involved in neurite formation. J.Cell Biol. 1997;139:951-961.

30. Konopaske GT, Subburaju S, Coyle JT, Benes FM. Altered prefrontal cortical MARCKS and PPP1R9A mRNA expression in schizophrenia and bipolar disorder. Schizophr.Res. 2015;164:100-108.

31. Finalet FJ, Rouhigharabaei L, Urbankova H et al. Integrative genomic and transcriptomic analysis identified candidate genes implicated in the pathogenesis of hepatosplenic T-cell lymphoma. PLoS.One. 2014;9:e102977.

32. Grass S, Preuss KD, Ahlgrimm M et al. Association of a dominantly inherited hyperphosphorylated paraprotein target with sporadic and familial multiple myeloma and monoclonal gammopathy of undetermined significance: a case-control study. Lancet Oncol. 2009;10:950-956.

598 33. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ.
599 Citrulline is an essential constituent of antigenic determinants recognized by
600 rheumatoid arthritis-specific autoantibodies. J.Clin.Invest 1998;101:273-281.

601 34. Thurner L, Fadle N, Regitz E et al. The molecular basis for development of
602 proinflammatory autoantibodies to progranulin. J.Autoimmun. 2015;61:17-28.

603 35. Doyle HA, Mamula MJ. Autoantigenesis: the evolution of protein modifications in
604 autoimmune disease. Curr.Opin.Immunol. 2012;24:112-118.

605 36. Thurner, L., Preuss, K. D., Fadle, N., Regitz, E., Klemm, P., Zaks, M., Kemele, M.,
606 Hasenfus, A., Csernok, E., Gross, W. L., Pasquali, J. L., Martin, T., Bohle, R. M., and
607 Pfreundschuh, M. Progranulin antibodies in autoimmune diseases. Journal of
608 autoimmunity in press. 2012.

609 37. Grass S, Preuss KD, Wikowicz A et al. Hyperphosphorylated paratarg-7: a new
610 molecularly defined risk factor for monoclonal gammopathy of undetermined
611 significance of the IgM type and Waldenstrom macroglobulinemia. Blood
612 2011;117:2918-2923.

613 38. Neumann F, Pfreundschuh M, Preuss KD et al. CD4(+) T cells in chronic autoantigenic
614 stimulation in MGUS, multiple myeloma and Waldenstrom's macroglobulinemia.
615 Int.J.Cancer 2015;137:1076-1084.

616 39. Montesinos-Rongen M, Schmitz R, Courts C et al. Absence of immunoglobulin class
617 switch in primary lymphomas of the central nervous system. Am.J.Pathol.
618 2005;166:1773-1779.

619 40. Torchia J, Weiskopf K, Levy R. Targeting lymphoma with precision using semisynthetic
620 anti-idiotype peptibodies. Proc.Natl.Acad.Sci.U.S.A 2016;113:5376-5381.

621 41. Ellebrecht CT, Bhoj VG, Nace A et al. Reengineering chimeric antigen receptor T cells
622 for targeted therapy of autoimmune disease. Science 2016;353:179-184.

Tables:

Table 1: Titers of SAMD14/neurabin-I antibodies in Serum/CSF pairs.

Case Serum/CSF	Anti-SAMD14/Neurabin-I-Serum titers	Anti-SAMD14/Neurabin-I-CSF titers
1	1:1600 – 1:3200	1:1600
2	1:3200	1:1600
5	1:1600	1:1600
10	1:800 -1:1600	1:800
14	1:1600	1:1600
15	1:1600	1:800
19	1:1600	1:800
21	1:1600	1:800

Figure Legends:

Figure 1: SAMD14 and neurabin-I as target antigen of recombinant PCNSL-derived B-cell receptors. a: ELISA of SAMD14 reactivity of PCNSL-derived recombinant B-cell receptors. The columns represent adsorbance at OD 490nm. **b: Western blots of PCNSL tissue lysates using SAMD14-specific recombinant BCR.** Lane 1: PCNSL tissue from patient #3 with a BCR not reacting with SAMD14; lane 2: PCNSL tissue from patient #6 with SAMD14 reactive BCR. Two proteins, one at 150kDa (above) and at 45kDa (bottom) of PCNSL are targeted by the recombinant BCR, with both antigens from patient #6 having a higher molecular weight compared to the antigens from patient #3. **c: Identification of neurabin-I as the second antigen.** Western blot of PNCSL tissue from patient #3: lane 1: the SAMD14 specific recombinant BCR targets proteins at 130kDa and 40kDa; lane 2: stripped Western blot for reprobing with a commercial antibody directed against the N-terminus of neurabin-I, identified the 130 kDa band as neurabin-I. **d: 2nd isoform of SAMD14 / neurabin-I in patients with SAMD14/neurabin-I-reactive lymphoma BCR.** Western blots of PNCSL tissues from patients with SAMD-14/neurabin-I specific

lymphoma cell BCRs (lanes II, III, IV and V) showed a higher molecular weight than from patients with BCR specificities other than SAMD-14/neurabin-I (lanes I, VI and VII), while bands for progranulin were not different. **E: ELISA for neurabin-I reactivity of individual PCNSL-derived recombinant BCRs**. The columns represent adsorbance at OD 490nm consistent with reactivity of the respective recombinant BCRs against neurabin-I.

Figure 2: N-Hyperglycosyation of SAMD14 and neurabin-I in PCNSL. a: N-hyperglycosylation of SAMD14 and neurabin-I in patients with SAMD14/neurabin-I-reactive lymphoma BCRs. Deglycosylation of SAMD14 and neurabin-1 from PCNSL tissue with a panel of endo- or exoglycosidases (PNGase-F, O-glycosidase, neuraminidase, ß-n-acetylglycosidase and galactosidase). Top: Lane 1: untreated PCNSL tissue from patient #3 (without SAMD14 reactive lymphoma BCR); lane 2: PNGase-F treated PCNSL tissue from #3. Lane 3: untreated PCNSL tissue from a patient #6 (with SAMD14 reactive lymphoma BCR); lane 4: PNGase-F treated PCNSL tissue from #6. Lane 5: O-glycosidase treated PCNSL tissue from #3; lane 6: O-glycosidase treated PCNSL tissue from #6. Lane 7: α-2(3,6,8,9)-neuraminidase treated PCNSL tissue from #3; lane 8: α-2(3,6,8,9)-neuraminidase treated PCNSL tissue from #6. Bottom: Lane 1: untreated PCNSL tissue from patient #3; lane 2: PNGase-F treated PCNSL tissue from #3. Lane 3: untreated PCNSL tissue from #6; lane 4: PNGase-F treated PCNSL tissue from patient #6. Lane 5: β-N-acetylglucosaminidase treated PCNSL tissue from #3; lane 6: β-N-acetylglucosaminidase treated PCNSL tissue from #6. Lane 7: β-1→4-galactosidase treated PCNSL tissue from #3; lane 8: β-1→4-galactosidase treated PCNSL tissue from #6. The treatment with PNGase-F and β-N-acetylglucosaminidase resulted in the disappearance of the differences in molecular weights of both antigens from patients #3 and #6. **b: Deglycosylation of SAMD14 and neurabin-1 from PCNSL tissue only with ß-n-acetylglycosidase.** The treatment with β-N-acetylglucosaminidase resulted in the disappearance of the differences in molecular weights of both SAMD14 and neurabin-I from patients #3 and #6 **c: Identification of the hyperglycosylated Asn residues.** Western blots of several constructs of SAMD14 and neurabin-I with different mutants of N-glycosylation sites expressed in LCLs derived from patients with and without hyperglycosylated SAMD14 and

676 neurabin-I. Anti-FLAG antibodies were used as primary antibodies. These Western
677 blots showed the disappearance of the gain in molecular weight for the point-
678 mutated, full-length proteins N339Q SAMD14 and N1277Q neurabin-I expressed in
679 LCLs of PCNSL patients with SAMD14/neurabin-I antibodies. d: **Identification of the**
680 **hyperglycosylated Asn residues.** Western blots of several constructs of SAMD14
681 and neurabin-I with different mutants of N-glycosylation sites expressed in LCLs
682 derived from patients with and without hyperglycosylated SAMD14 and neurabin-I.
683 Instead Anti-FLAG primary antibody recombinant SAMD14/neurabin-I-specific Fab
684 was used as primary antibody. These Western blots show double bands as they
685 unspecifically stain for both wild-type SAMD14 (hyperglycosylated) and non-
686 hyperglycosylated N339Q SAMD14 and both for wild-type neurabin-I
687 (hyperglycosylated) and N1277Q neurabin-I.

688

689 **Figure 3: BCR pathway activation and proliferation induced by BCR-specific**
690 **antigens. a: Activation of the BCR signaling pathway.** The Western blot analysis
691 of the BCR signaling pathway shows a strong activation by addition of SAMD14 in
692 transfected TMD8 and U2932 cells expressing a SAMD14/neurabin-I-reactive BCR or
693 by addition of anti-IgM. The addition of the cognate antigens or anti-IgM antibody
694 results in the upregulation of pTyr525/526 SYK, pTyr96 BLNK, pTyr759 PLCγ2 and
695 pTyr223 BTK and led to an overexpression of c-Myc. **b:**. **Proliferation induction of**
696 **aggressive lymphoma cell lines by BCR-specific antigens.** The columns
697 (formazan at an OD of 450 nm) represent the proliferation of cells measured by
698 reduction of tetrazolium salt to colored formazan by viable cells (EZ4U assay).
699 Addition of epitope containing region of neurabin-I resulted in a significant increase in
700 proliferation of TMD8 and U2932 cells expressing SAMD14/neurabin-I-reactive
701 BCRs.

702

703 **Figure 4: Specific killing of aggressive lymphoma cells using BCR-**
704 **antigen/toxin constructs. a: LDH-release assay.** Percent specific lysis of non-
705 transfected OCI-Ly3 cells or OCI-LY3 cells transfected to express either MAZ-specific
706 or SAM-specific BCRs after incubation with 5 µg/mL neurabin-I/ETA or MAZ/ETA,
707 respectively,for 24h. **b: Trypan blue exclusion assay.** Cell viability of transfected

OCI-LY3 cells expressing SAMD14/neurabin-I-specific BCRs or non-transfected OCI-Ly3 cells after incubation with 2 µg/mL neurabin-I/ETA or MAZ/ETA was determined by trypan blue staining after 24h and 48h showing the time-course effect. **c: Dose dependency of cytotoxicity determined by LDH-release assay.** Percent specific lysis of transfected TMD8 cells either Doxycyclin-induced or non-induced after incubation with different concentrations 1.25 – 10 µg/mL of neurabin-I/ETA' or LRPAP1/ETA' immunotoxins for 24h showing a dose-dependent effect. **d: Annexin-V assay.** Characterization of transfected TMD8 and U2932 cells with either Doxycyclin-induced expression of SAMD14/neurabin-I reactive BCR or non-induced, non-expressing recombinant BCRs by annexin-V and PI staining after 24h cultivation in the presence of SAM/ETA, LRPAP1/ETA or Staurosporin.

Figures

Figure 1

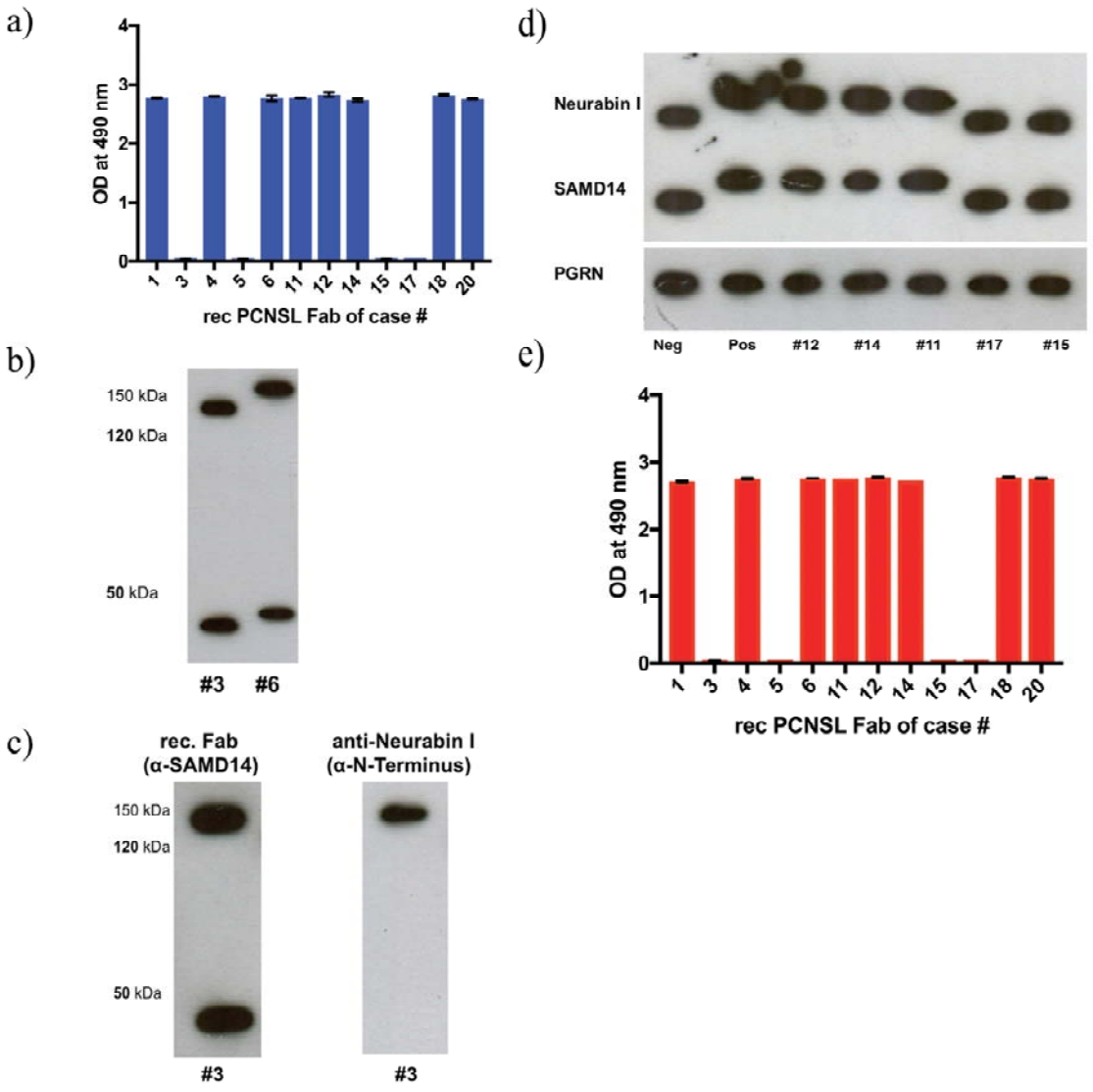


Figure 2

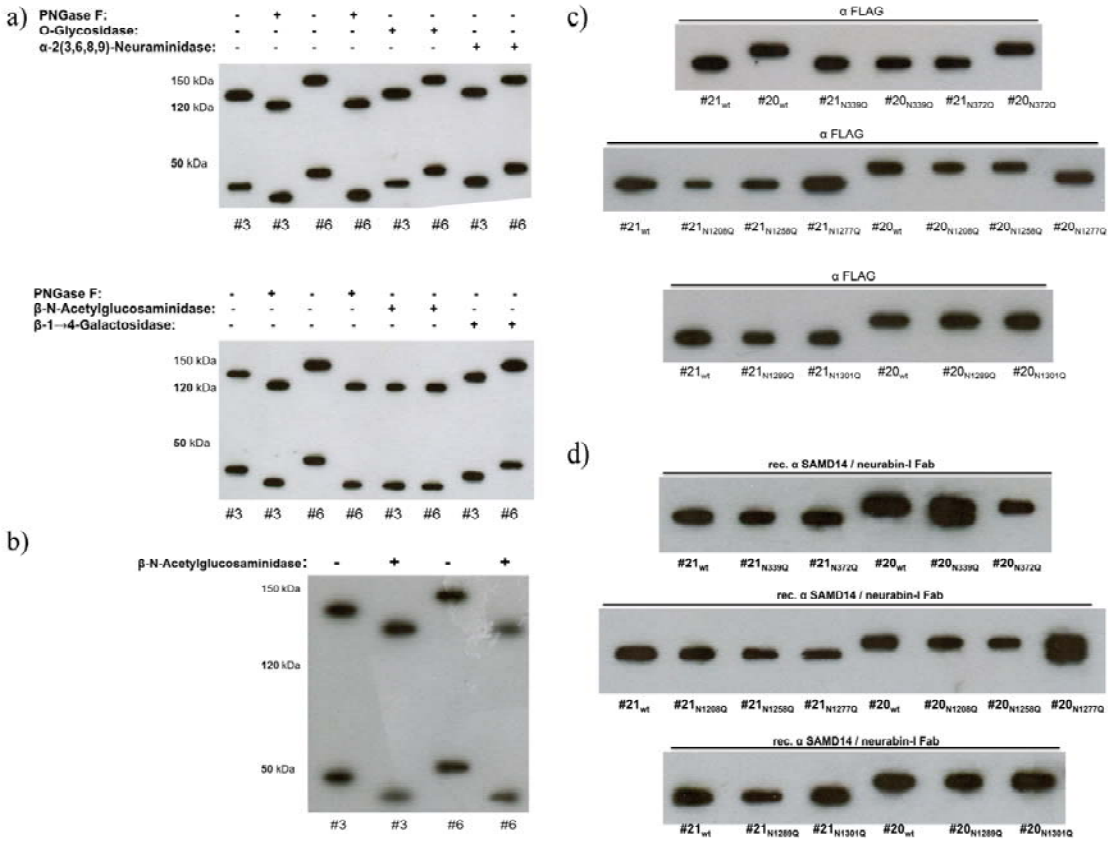
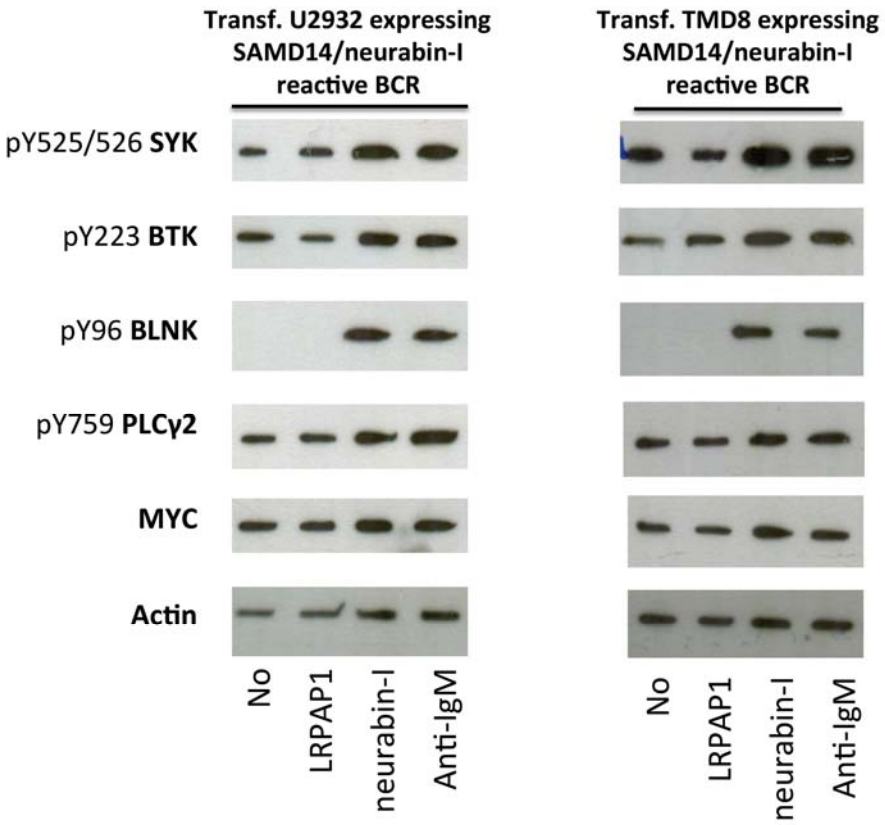

a)
PNGase F:
O-Glycosidase:
α-2(3,6,8,9)-Neuraminidase:
150 kDa
120 kDa
50 kDa
#3 #3 #6 #6 #3 #6 #3 #6
PNGase F:
β-N-Acetylglucosaminidase:
β-1→4-Galactosidase:
150 kDa
120 kDa
50 kDa
#3 #3 #6 #6 #3 #6 #3 #6
b)
β-N-Acetylglucosaminidase:
150 kDa
120 kDa
50 kDa
#3 #3 #6 #6
c)
α FLAG
#21wt #20wt #21N339Q #20N339Q #21N372Q #20N372Q
α FLAG
#21wt #21N1208Q #21N1258Q #21N1277Q #20wt #20N1208Q #20N1258Q #20N1277Q
α FLAG
#21wt #21N1289Q #21N1301Q #20wt #20N1289Q #20N1301Q
d)
rec. α SAMD14 / neurabin-I Fab
#21wt #21N339Q #21N372Q #20wt #20N339Q #20N372Q
rec. α SAMD14 / neurabin-I Fab
#21wt #21N1208Q #21N1258Q #21N1277Q #20wt #20N1208Q #20N1258Q #20N1277Q
rec. α SAMD14 / neurabin-I Fab
#21wt #21N1289Q #21N1301Q #20wt #20N1289Q #20N1301Q

Figure 3

a)


Transf. U2932 expressing SAMD14/neurabin-I reactive BCR
Transf. TMD8 expressing SAMD14/neurabin-I reactive BCR
pY525/526 SYK
pY223 BTK
pY96 BLNK
pY759 PLCγ2
MYC
Actin
No
LRPAP1
neurabin-I
Anti-IgM

b)

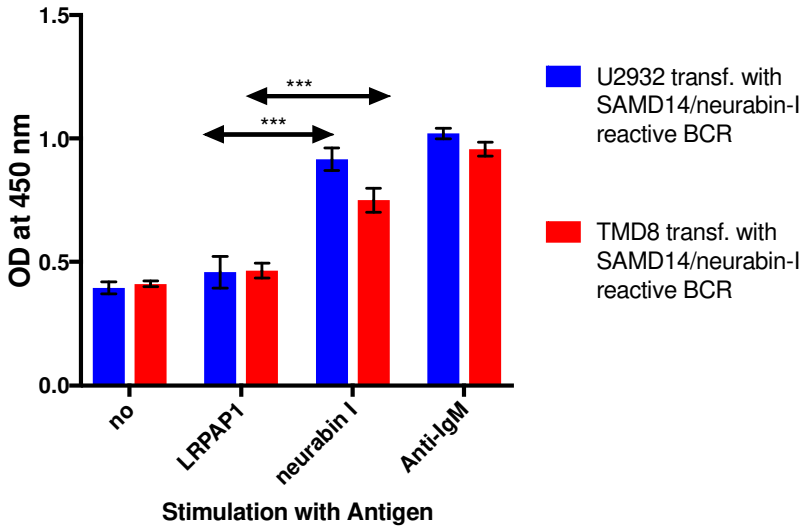
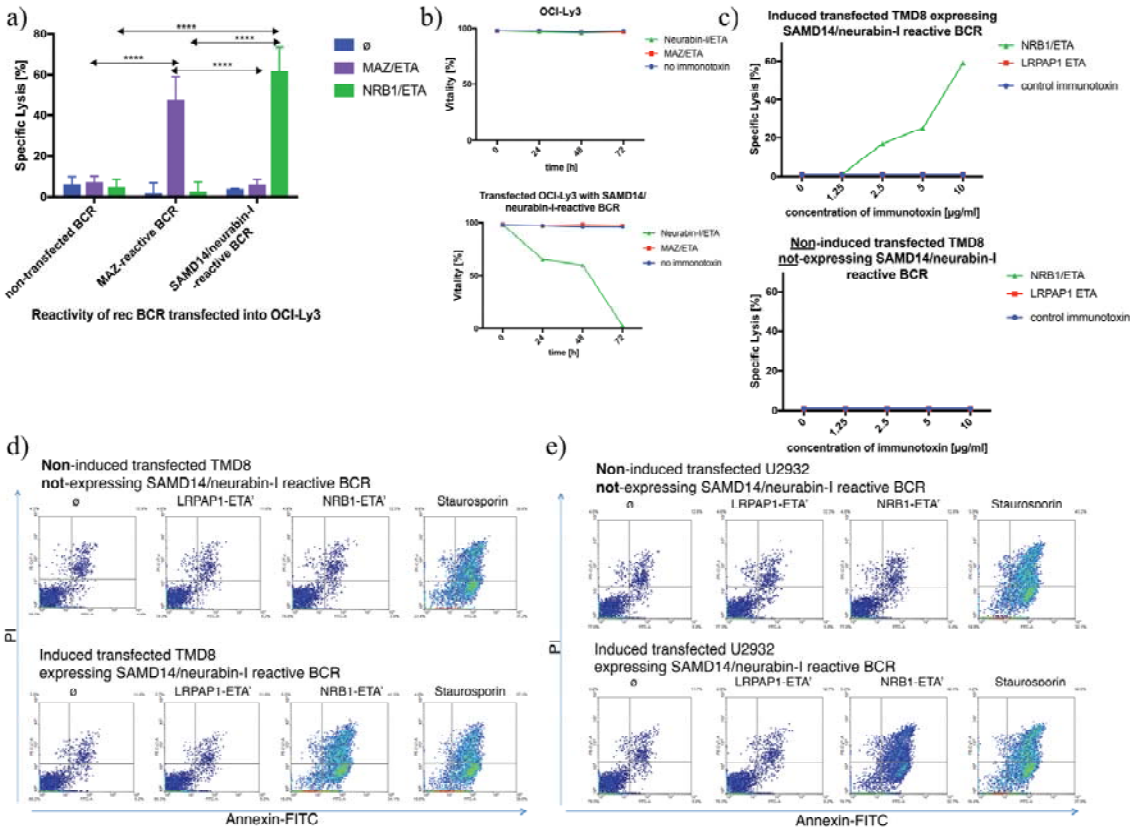

OD at 450 nm
1.5
1.0
0.5
0.0
***
***
no
LRPAP1
neurabin I
Anti-IgM
Stimulation with Antigen
U2932 transf. with SAMD14/neurabin-I reactive BCR
TMD8 transf. with SAMD14/neurabin-I reactive BCR

Figure 4


a)
Specific Lysis [%]
ø
MAZ/ETA
NRB1/ETA
non-transfected BCR
MAZ-reactive BCR
SAMD14/neurabin-I -reactive BCR
Reactivity of rec BCR transfected into OCI-Ly3
b)
OCI-Ly3
Vitality [%]
time [h]
Neurabin-I/ETA
MAZ/ETA
no immunotoxin
Transfected OCI-Ly3 with SAMD14/ neurabin-I-reactive BCR
c)
Induced transfected TMD8 expressing SAMD14/neurabin-I reactive BCR
NRB1/ETA
LRPAP1 ETA
control immunotoxin
concentration of immunotoxin [µg/ml]
Non-induced transfected TMD8 not-expressing SAMD14/neurabin-I reactive BCR
d)
Non-induced transfected TMD8
not-expressing SAMD14/neurabin-I reactive BCR
ø
LRPAP1-ETA'
NRB1-ETA'
Staurosporin
Induced transfected TMD8
expressing SAMD14/neurabin-I reactive BCR
PI
Annexin-FITC
e)
Non-induced transfected U2932
not-expressing SAMD14/neurabin-I reactive BCR
Induced transfected U2932
expressing SAMD14/neurabin-I reactive BCR